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Physiological Responses of *Thalassia testudinum* and *Ruppia maritima* to Experimental Salinity Levels

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By

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ABSTRACT

Thalassia testudinum, a stenohaline seagrass species, and *Ruppia maritima*, a euryhaline submerged aquatic vegetation species, were subjected to the same seven salinity levels (0 – 60) in a controlled environment. The response variables examined were the occurrence of leaf discoloration, plant growth rates, photosynthetic characteristics of blade segments (Pmax, respiration, alpha, and I_k), and osmolality changes within the plant tissues. These response variables were measured at exposure times of one, seven, and 28 days.

Greater than 75% leaf discoloration occurred in *Thalassia testudinum* blades placed in 0, and 60 psu, while *Ruppia maritima* blades only became severely discolored in 60 psu. Plant growth rates were highest in 40 psu for *T. testudinum* and 20 psu for R. *maritima*. Pmax for both species was somewhat affected by salinity changes, but the plants did not appear to be photosynthetically compromised in their "optimal" ranges over time. Salinity effects on photosynthesis were less pronounced in *R. maritima* than in *T. testudinum*, which would be expected when comparing a euryhaline species to a

stenohaline species. Both intercellular and intracellular osmolality showed a pattern of increase or decrease as the treatment salinities were altered from ambient levels (30 psu for *T. testudinum* and 20 psu for *R. maritima*). After one day of exposure to a new treatment salinity, the intercellular osmolality had changed significantly from ambient value, with a second shift, occurring mostly in the salinity extremes, for both seagrass species. This second shift is most likely due to the fact that at the extremes, the plants are being compromised.

Changes in these physical and physiological responses indicate that significant increases and decreases in ambient salinity levels are initially stressful for both species. Both seagrass species had an optimal salinity as well as a range of salinities in which the long-term physiological stresses did not cause tissue death. *Thalassia testudinum* had the fewest stress responses in 40 psu, with an optimal range of 20 – 40 psu. *Ruppia maritima* had the fewest stress responses in 20 psu (growth salinity) with an optimal range of 0 – 40 psu. In this study, neither species was able to survive for 28 days in 60 psu (at which point the plants had been out of their respective optimal salinities for at least 42 days).

Chapter 1: Responses of *Thalassia testudinum* to salinity variations

Introduction

Seagrasses are submerged aquatic angiosperms that are vital components of coastal and estuarine ecosystems throughout the world. These plants create, as well as occupy, important niches in shallow water environments. They are not only highly productive members of nearshore ecosystems, but their complex structure provides habitat, food, substrate, and protection for many different types of fish and invertebrates (Zieman, 1987). Seagrasses influence the dynamics of the areas they inhabit by affecting sedimentation, water chemical balance, and water movement in their immediate vicinity (Koch, 2001). Since seagrasses grow completely submerged, they are affected by a number of environmental factors; among these, salinity appears to play a major role in submersed aquatic vegetation community distribution, composition, and relative abundance (Zieman, 1982; Livingston, 1987; Montague and Ley, 1993), as well as seagrass survival, growth, and production (Walker and McComb, 1990).

Water-management practices can change inshore marine communities by altering natural freshwater discharge rates from inland areas. Interference with freshwater flows affects salinity patterns in coastal areas. In southern Florida, the creation of canals and water-control structures has disrupted freshwater flow into nearshore areas (Montague and Ley, 1993). Freshwater enters Florida Bay

in three ways: overland sheet flow, local rainfall, and river or canal flow from manipulations of South Florida Water Management District's canal system. Water management began in the Florida Everglades in the 1800's and continued through the 1960's (Smith et al., 1989; McPherson and Halley, 1996). As much as 70% of the historical freshwater flow through the Everglades into Florida Bay has been diverted for human use by water management practices (Smith et al., 1989). This diversion, and resultant changes in the historical distribution of freshwater runoff caused an increase in mean salinity, as well as an increase in the frequency and amplitude of salinity fluctuations in Florida Bay (Tilmant et al., 1987; Smith et al., 1989; Brewster-Wingard and Ishman, 1999). These fluctuating salinities can alter biota distribution and abundance in Florida Bay and other coastal areas (Montague and Ley, 1993).

In addition to water-management practices, many other factors are also involved in salinity fluctuations in Florida Bay. Depending on local rainfall, parts of the bay alternate between hypersaline and brackish conditions (Robblee et al., 1991). Other factors affecting the salinity in Florida Bay include evaporation and saltwater influx from the Gulf of Mexico and the Atlantic (Smith et al., 1989; Rudnick, 1999). Heavy rainfall substantially increases freshwater input to the bay, resulting in lowered salinities. Lack of rainfall reduces local freshwater inputs while concomitantly increasing human demand for freshwater and reducing the availability of runoff from upland areas. Often, environmental and anthropogenic alterations of freshwater influx into Florida Bay synergistically increase salinity variations in the Bay. Reduction of freshwater flow to Florida

Bay causes significant salinity increases in some areas of the bay (Durako et al., 1994).

Seagrass habitat can be altered by freshwater diversion and the resultant alteration of water quality. Studies in Apalachee Bay, Florida showed that relatively minor changes in water-quality could alter seagrass distributions and productivities (Livingston, 1987). Livingston (1984) concluded that freshwater influx into estuarine areas could degrade seagrass beds due to salinity fluctuations and other water-quality changes. Zieman (1982) proposed that changes in Florida Bay seagrass distributions might be linked to changes in salinity caused by altered freshwater inputs. Other research shows that salinity fluctuations could cause alterations in both the distributions and total abundances of benthic vegetation (Montague and Ley, 1993; Fourqurean et al., 2003).

Near the mouth of the Mississippi River, increased freshwater diversion into seagrass beds has had a detrimental effect on many species of submerged aquatic vegetation (SAV) (Eleuterius and Miller, 1976). Adams et al. (1992) determined that salinity influences species distribution and composition within submerged macrophyte communities. An increase of freshwater runoff into an area may favor estuarine characteristics over marine conditions in that area (Eleuterius and Miller, 1976). Salinity fluctuations in an estuarine area may favor the growth of some euryhaline seagrass species, *Ruppia maritima* L. (widgeon grass), for example (Hoese, 1960), and inhibit the growth of other seagrasses with narrower salinity requirements, such as *Thalassia testudinum* Banks. ex König (turtle grass).

Seagrasses have become adapted to high external osmotic pressures and can to some degree avoid the toxic effects of high salinities (Munns et al., 1983). The effects of salinity on physiological processes of seagrasses, such as photosynthetic responses, have been investigated by many researchers (Ogata and Matsui, 1965; Zieman, 1974; Kerr and Strother, 1985; Murphy et al., 2003). Dilution or concentration of formerly full-strength seawater (31 psu) causes changes in growth and photosynthetic rates of seagrasses (Mc Millan and Mosely, 1967; Hammer, 1968; Biebel and McRoy, 1971; Zieman, 1975). Environmental stressors, such as salinity fluctuations, decrease the maximum photosynthetic rates of some seagrass species within the same available irradiance levels (Williams and McRoy, 1982).

Seagrasses increase plant-tissue or plant-sap osmolality with an increase in salinity (Brock, 1981; Van Digglen et al., 1987; Murphy et al., 2003). The ability of halophytes to tolerate high salinity is directly related to osmoregulation, by such means as proline and soluble carbohydrate accumulation within the plants and other methods, such as active ion pumping (Brock, 1981; Jagels, 1983; Jagels and Barnabas, 1989; Murphy et al., 2003). Studies on the after-effects of salinity fluctuations show that some species recover from hypersaline conditions when the salinity is reduced, but other species do not recover even when salinity is lowered to the control level (Adams and Bate, 1994).

Thalassia testudinum is the dominant seagrass species in the tropical and subtropical waters of the western Atlantic, the Caribbean region, and Florida Bay. It is considered to be a stenohaline marine species, with optimum growth

occurring at salinities between 24 and 35 psu (Phillips, 1960; den Hartog, 1970; Zieman et al., 1989). *T. testudinum* can most commonly be found at depths of less than 12 meters and often forms extensive beds in shallow water (Zieman, 1987). Because seagrasses are a major component of coastal and estuarine ecosystems and fill multiple roles in the established tropic dynamics of these systems, the evaluation of the impact of salinity alterations and fluctuations in an area such as Florida Bay is critical.

Salinity is an important issue in Florida Bay because it has been, and continues to be affected by humans through water control practices. Changes proposed to the South Florida C-111 canal system will provide more historically natural sheet flow to Florida Bay. One way that this will be achieved is by reducing point sources of freshwater discharge into estuarine systems of Florida Bay through the C-111. Also in progress are projects to restore historical tidal flow that was eliminated in the early 1900's during the construction of Flagler's railroad through the Everglades, linking the Keys to the mainland. These projects would increase exchange between the waters of Florida Bay and the Atlantic in order to hypothetically "significantly improve water quality, benthic floral and faunal communities, larval distribution of both recreational and commercial species and the overall hydrology of Florida Bay" (CERP website). Together these projects will affect the salinity patterns in Florida Bay and ultimately these changes in freshwater flow will affect the benthic vegetation (Fourqurean et al., 2003).

The present study was designed to determine the physiological and physical responses of *Thalassia testudinum* to different salinities ranging from 0 to 60 psu in a controlled environment. The response variables that were used to determine the "stress" in seagrass associated with each salinity level included changes in photosynthetic responses, osmolality changes within plant blades, changes in plant growth and leaf turnover rates, and visual estimates of leaf color change from green to brown, which is evidence of tissue death. These variables were used to determine upper and lower salinity tolerance thresholds for *T. testudinum* under laboratory conditions, and to assess the amount of "stress" associated with each experimental salinity level between 0 and 60 over 1 - 28 days exposure time.

Materials and methods:

Thalassia testudinum seedlings were grown from fruits collected along the shore of Biscayne Bay in Matheson Hammock Park, Miami, Florida (Fig. 1). The fruits were collected in August 1995, and were found either floating offshore, or buried within the high tide wrack among mangrove trees fringing the shoreline. Seeds were removed from dehisced fruits and allowed to float freely in a115 L (30 gal) aquarium filled with Instant Ocean © brand synthetic seawater (IO) at 30 psu until they began to grow roots, which was approximately three to five weeks. The seedlings were then planted in 2x2x5" plastic pots filled with washed aragonite shell hash and then placed in aquaria containing IO at 30 psu. All IO was prepared using tap water. The use of tap water in sea grass cultures has

been established by other researchers (McMillan and Mosely, 1967; McMillan, 1980; Mitchell, 1987). All plants were grown in 115 L aquaria filled with IO at a salinity of 30 psu at 25 - 28° C with a 12-hour photoperiod at a light intensity of 40 - 100 µmoles quanta m^{-2} s^{-1} (measured at the front and back of the experimental tanks) until needed for salinity experiments. Four 40-watt full-spectrum fluorescent tubes provided light to each pair of aquaria.

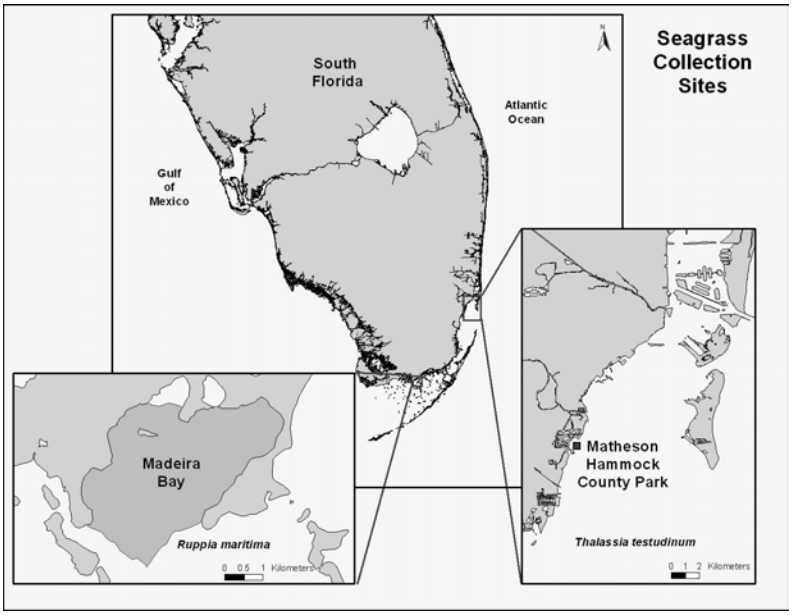

Fig. 1. Map of the collection sites for *Thalassia testudinum* fruits and *Ruppia maritima* plants used in this experimentation.

Water was added regularly to replace water lost due to evaporation, and air stones provided aeration and water movement within the tanks. The experimental units were placed in three rows, which were rotated weekly within the aquaria to compensate for the variation in light levels at the front and back of the tanks.

The *Thalassia testudinum* seedlings were 19 months old when salinity experimentation was begun. The plants used in the salinity tolerance experiments were randomly chosen from the holding aquaria, and placed in treatment aquaria. A separate aquarium was used for each salinity treatment between 0 and 60 psu. To reach test salinities, the original growth salinity of 30 psu was increased or decreased in 10 psu increments until the new salinities were reached. The plants were allowed to acclimate to each 10 psu change for one week before the next salinity adjustment. Each experimental tank contained eight *T. testudinum* seedlings, which were placed in a single row. Three of these plants were used for determination of plant growth rate in the treatment salinities. Four of the other plants were used for physical and physiological experimentation at each salinity level; one seedling was a spare to be sampled if necessary. The four experimental units were each identified by colored flags (green, blue, red, and white) to insure repeated measures involved the same seedling at each time interval. A repeated measures system was used such that a single blade from each replicate was used each of the sampling days, and the same seedling was used throughout the 28-day treatment. If the youngest blade was not mature enough, the next youngest was used.

Plant responses to salinity levels were monitored using visual analysis of tissue death of the four replicate plants, plant growth rates, photosynthesis (P) vs. irradiance (E) relationships, and measurement of osmolality in leaf tissues. These response variables were measured at exposure times of one, seven, and twenty-eight days in the test salinity. The individual plants were also monitored

initially and then weekly for leaf tissue “stress” response as observed in blade color change from green (healthy) to brown (dead) (Table 1).

PLANT TISSUE DISCOLORATION SCALE
0 = 100% green, no discoloration
1 = less than 5% discoloration
2 = 5 to <25% discoloration
3 = 25 to <50% discoloration
4 = 50 to <75% discoloration
5 = 75% or greater discoloration

Table 1. Plant discoloration scale used to rate plant color based on percentage of replicate that had become discolored (chlorotic, yellow, or brown), and thus essentially non-functioning. This was used as a visual indicator of a stress response to the treatment salinities.

Seedling growth was measured using leaf-marking-based productivity measurements as outlined by Patriquin (1973) and Zieman (1974). The plants were leaf punched using a 21 gauge needle at t = 7 days exposure in the treatment salinity, and harvested at t = 21 days. These data provided information about turnover rates and production per day. Dry weight production per day was calculated as the increment in dry weight production per day of each leaf; leaf area production is the total area of new material produced per day; and leaf turnover is the total area of new material/14 days/ total leaf area of the blade.

A 3 cm segment from the base of the youngest, fully developed turtle-grass blade was taken from each replicate plant to be used in response measurements at 1, 7, and 28 days exposure. The first half-centimeter segment

from the base was used for photosynthesis vs. irradiance measurements, the second centimeter was used in osmolality determination, and the third centimeter was dried at 60 C° to a constant weight and used for dry weight measurements. After the P vs. E runs were completed, the leaf material used was frozen and used later for chlorophyll extraction with 90% acetone. The chlorophyll analysis was done by measuring light absorbance in the 280-800 nm range. Chlorophyll *a* and *b* were then calculated using the dichromatic formulae of Jeffrey and Humphrey (1975).

Photosynthesis was measured as a change in concentration of dissolved oxygen in a closed system, as outlined by Beer et al. (1977) and Durako and Kuss (1994). All P vs. E experiments were run at 25 C°. A Hansatech DW/1 Clark-type oxygen-electrode system was used to measure net photosynthesis for the four replicate plants in each salinity treatment. A 1-cm long leaf segment was placed in a closed chamber filled with 2.5 ml of nitrogen (N_2) sparged seawater with the appropriate treatment salinity. The seawater was bubbled with N_2 to reduce O_2 concentrations to about 25% of saturation to both prevent the formation of gas bubbles during photosynthetic measurements and because photosynthetic capacity of marine angiosperms is reduced by elevated concentrations of dissolved O_2 (Downton et al., 1976). Mixing was provided by a magnetic stirring bar inside the chamber. This vigorous stirring, as reported by Bulthuis (1983), is required to establish equilibrium between the O_2 concentration of the seawater in the chamber and the O_2 concentration in the plant tissues and to minimize the effects of oxygen accumulation in the lacunae of the *Thalassia*

testudinum tissues. The chamber temperature was controlled by water from a refrigerated water bath being circulated through the outer jacket of the chamber. Light was provided by a Kodak ectographic slide projector with a 300-watt bulb. Light intensity was varied with neutral density filters placed between the projector and the plant chamber and was measured with a cosine-corrected quantum sensor connected to a model Li Cor datalogger (LI-1000), which measures in the photosynthetically active radiation (PAR = 400 - 700 nm) range of the spectrum.

Plant material in the chamber was allowed to equilibrate in the dark for ten minutes. After equilibration, the plants were subjected to twelve increasing light levels ranging from ≈10uE to ≈1000uE of PAR. A one-minute equilibration time for each light level was used before the initial O_2 readings were made. Another reading was taken two minutes later, and the del (Δ) value (the difference between these two readings) was used for oxygen-flux calculations.

All photosynthesis parameter values are expressed in μmoles O_2 mg^{-1} chl*a* h^{-1}. All Hansatech readings are net photosynthesis, with respiration being the initial dark readings. The gross values were obtained by adding dark respiration to all consecutive light-level O_2 concentration readings. The P vs. E data were used to calculate the following response variables: alpha, the initial slope of the regression line; Pmax, the light level at which maximum photosynthetic activity was reached; and I_K, the saturation irradiance, and is calculated Pmax/alpha. Respiration was calculated from the 2-minute Δ value recorded after the initial ten-minute dark-incubation period. The *Thalassia testudinum* P vs. E curves exhibited typical saturation kinetics, which were similar

to those, described by Jassby and Platt (1976). P vs. E response variables were calculated using a least-squares non-linear curve-fitting algorithm in Sigma Stat (Jandel Scientific, CA). The data were fitted to the hyperbolic tangent equation y = Pmax*tanh (alpha*x/Pmax), where y = O_2 flux and x = irradiance. All P vs. E curves were plotted as gross photosynthesis and as a function of the curve fit (Fig. 2).

Osmolality of *Thalassia testudinum* tissue samples was measured, as proposed by Tyerman (1982, method 1), using a Wescor Vapor Pressure Osmometer 5500C, which calculates solute concentration from sample vapor pressure compared to the vapor pressure of standard solutions. A 4.5 cm diameter tissue disc from each turtle-grass blade was used. The blades were punched, the tissue was blotted quickly to remove any surface water, and the tissue was immediately placed in the osmometer. Additional care was taken to reduce the effects of evaporation during handling by cutting the tissue discs while the plant blades were fully submerged.

The osmometer was calibrated against 290 and 1000 μmol kg^{-1} standards to encompass the full range of possible readings. The plant tissue was allowed to equilibrate inside the osmometer thermocouple chamber for 20 minutes before a reading was made. This 20-minute interval was experimentally determined as outlined by Tyerman (1982) for establishing tissue-chamber equilibrium (Fig. 3). The tissue sample was placed in the chamber, and an initial reading was taken. Readings were made every two minutes until a stable level was reached (20 minutes), after that, readings were taken every five minutes to check stability.

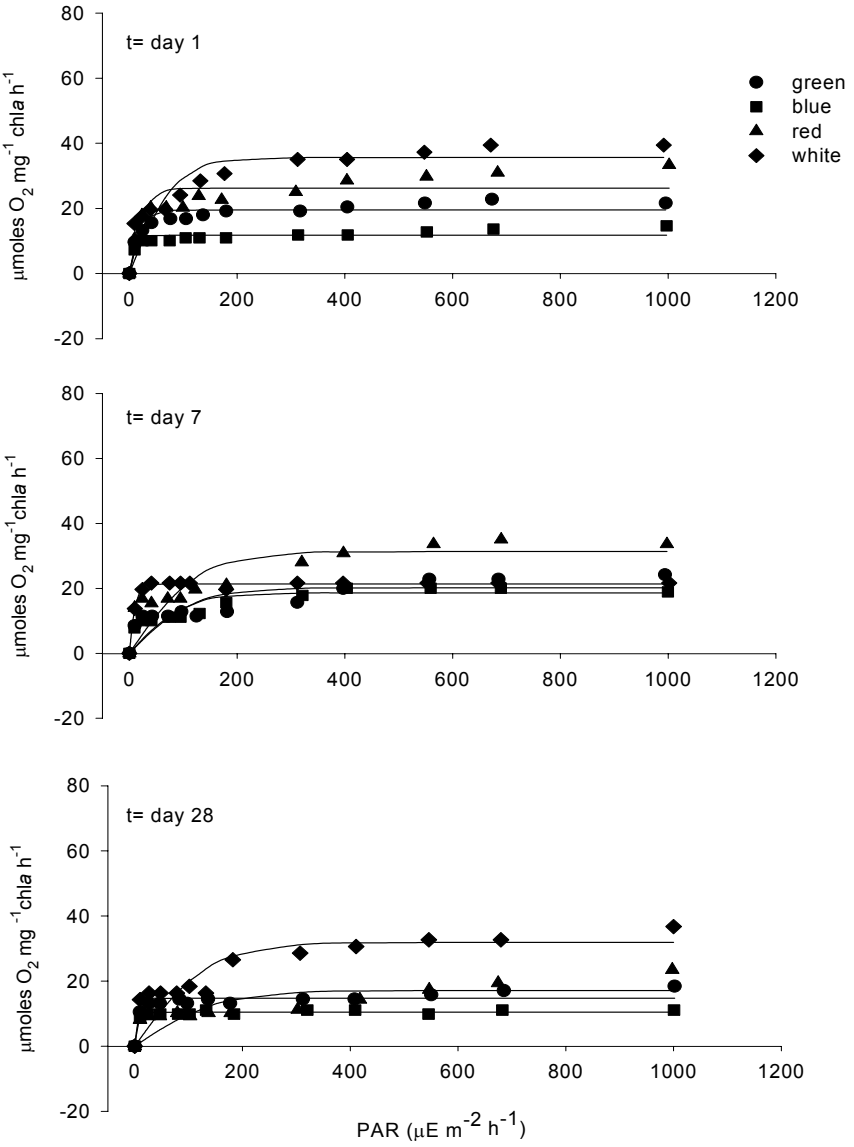

Fig. 2. Graph showing gross photosynthesis (scatter plots) of *Thalassia testudinum* plants in 20 psu Instant Ocean synthetic seawater at exposure times of 1,7, and 28 days. The data are also shown as a function of the hyperbolic tangent equation y = Pmax*tanh (alpha*x/Pmax) (spline curves).

Tyerman's protocol measures leaf-water potential and free cytoplasmic ions providing a measure of intercellular osmolality. After this initial reading was made, the tissue was frozen overnight to fracture internal membranes, and a second osmolality reading was taken. This second reading, using previously

frozen plant material, measures the intracellular osmotic pressure, including any vacuole and cytoplasmic ion concentrations.

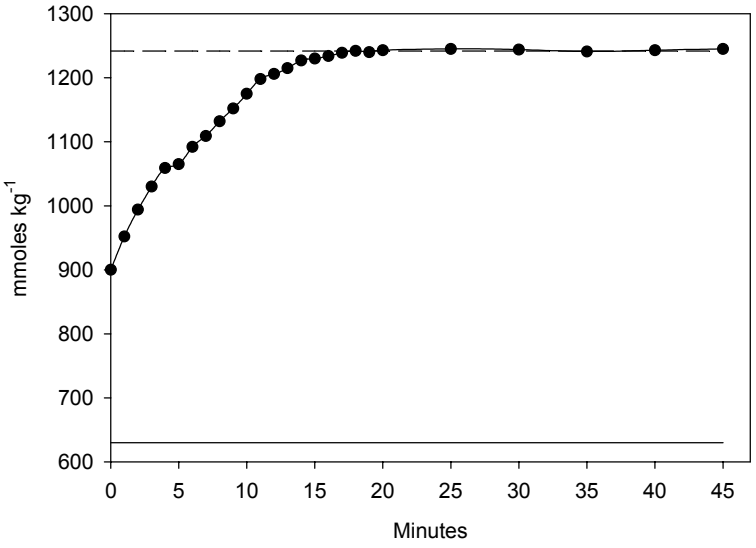

Fig. 3. Graph showing the establishment of tissue-chamber equilibrium time for *Thalassia testudinum* tissue at 30 psu (per Tyerman, 1982). The symbols represent osmolality measurements over time. The osmolality of Instant Ocean mixed to 30 psu is represented on the graph as a solid line, while the average intracellular osmolality of *T. testudinum* tissue at 30 psu and an exposure time of 1 day is represented as a dashed line.

The effects of salinity and exposure time on leaf color, growth rates, photosynthesis, and osmolality were assessed using linear regressions and two-way ANOVAs. All data sets were tested for normality and equality of variances using the Kolmogorov-Smirnov test ($p<0.001$). When both normality and equal variance passed, *post-hoc* comparisons were made using Student-Newman-Keuls method. In most cases, normality failed but variance equality passed. If no transformations could bring about normality, then two-way ANOVAs were performed to determine the effects of time and salinity on the original data. In

the cases where homogeneity also failed, and no transforms were successful, Kruskal-Wallace one-way ANOVA on ranks were performed. If significant differences were found through the ANOVAs, either Tukey's or Dunnett's pairwise multiple comparisons were made to determine specific statistical differences. All statistics were performed with SigmaStat software with a significance level of 95%.

Results:

Changes in leaf color

Thalassia testudinum plants placed in 20 - 40 psu showed no noticeable change in leaf color over time. Plants in 0, 10, 50, and 60 psu showed a decline in "healthy" leaf color after 1 week, with plants in 0 psu and 60 psu becoming completely brown (dead) by 3 weeks (Fig. 4).

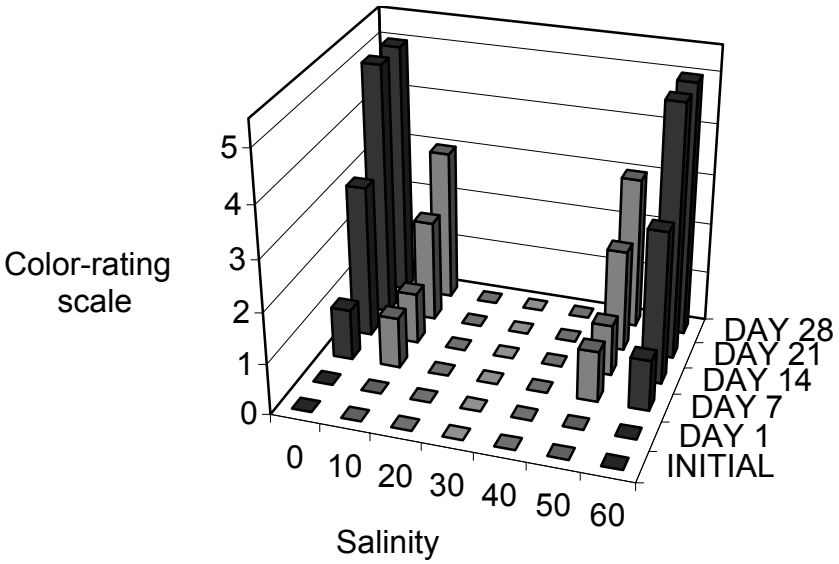

Fig. 4 *Thalassia testudinum* leaf color change observed weekly over the 28-day experimental period in salinities 0 – 60 psu. Color scores are based on a 0 - 5 rating scale, with 0 being 100% green and 5 being 100% brown.

Changes in leaf growth rates

The highest rates of leaf growth, as well as the fastest dry weight turnover rates, were seen in the 40 psu treatment (Figs. 5a, b, c). Production in blade length and weight per day were highest in 40 psu (0.37cm/1.35 g) and lowest in 0 psu (0.01cm/0.05 g). Rates of growth and leaf turnover decreased and increased respectively as salinity was moved in either direction from 40 psu.

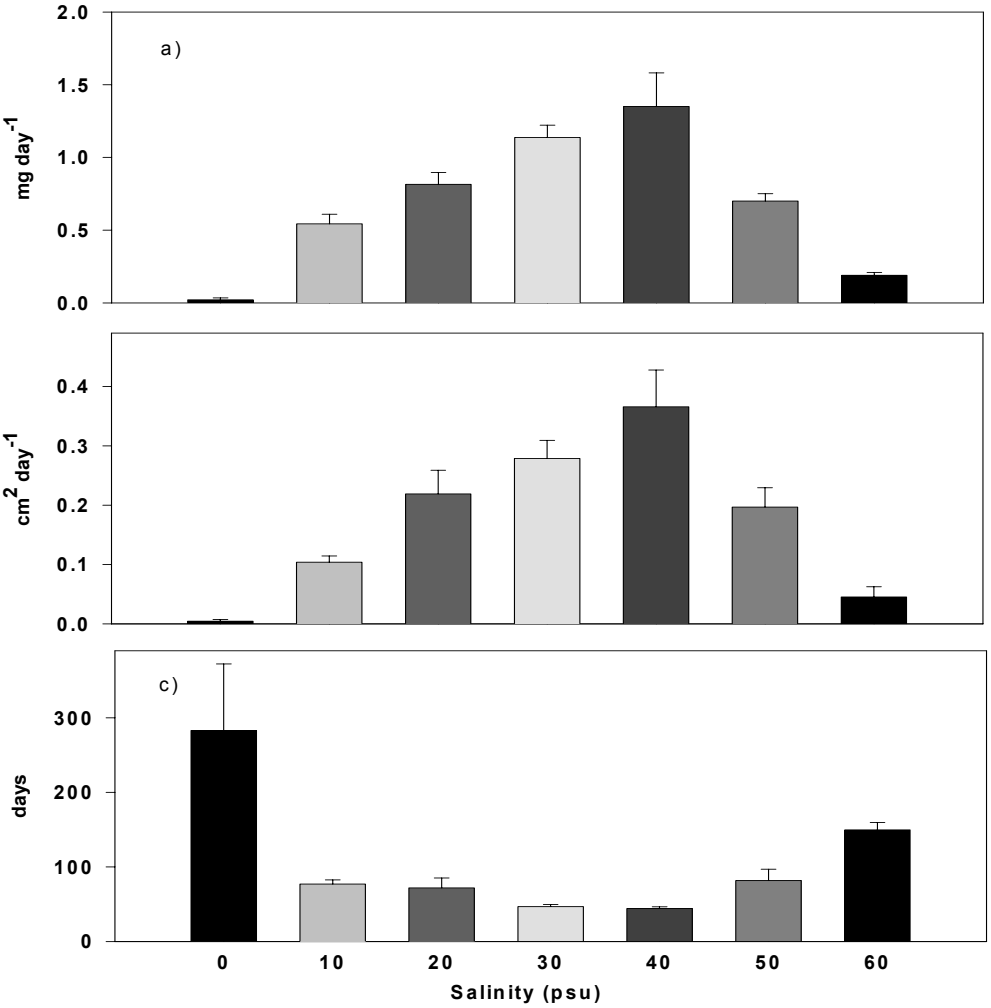

Fig. 5. *Thalassia testudinum* growth rates (mean ± standard error) from exposure time t = 7 days to t = 28 days in experimental salinities of 0 – 60 psu. Two measures were used to determine daily rates of growth a) mg dry weight produced and b) cm of blade material produced. These data were then used to calculate c) leaf turnover rates for each experimental salinity.

Dunn's method was used to perform a pairwise multiple comparison to isolate the differences in responses brought about by the treatment salinities. For all growth parameters, 20, 30, and 40 psu were most similar to one another, 10 and 50 psu were alike, and 0 and 60 varied most from the other treatment salinities.

Changes in photosynthetic characteristics

At the end of 1 day in the experimental salinities, plants in 30 psu exhibited the highest photosynthetic capacity as indicated by the highest Pmax and lowest respiration values. After 7 days and 28 days exposure, the highest average Pmax was seen in the 40 psu treatment, with decreasing values as salinity varied from this level in both directions. There was a significant salinity effect on Pmax values (Fig. 6a), but no exposure time effect. Tukey's test, used to compare the effects of different salinities, indicated that 20, 30, and 40 psu treatments were most alike, and these treatment levels appeared to have the least detrimental effect on Pmax values. Respiration showed no significant changes over exposure time or among salinity treatments (Fig. 6b). However, after 28 days exposure, the pattern of respiration versus salinity was the inverse of the pattern of Pmax versus salinity (compare Figs. 6a and b).

Changes in leaf tissue osmolality

Intercellular (fresh) osmolality changed in all salinities over time, with 20, 30, and 40 psu treatments showing the least variation throughout the month (Fig. 7a). Leaf osmolality values ranged from 375 μmol kg^{-1} (0 psu, day 7) to 2250 μmol kg^{-1} (50 psu, day 28).

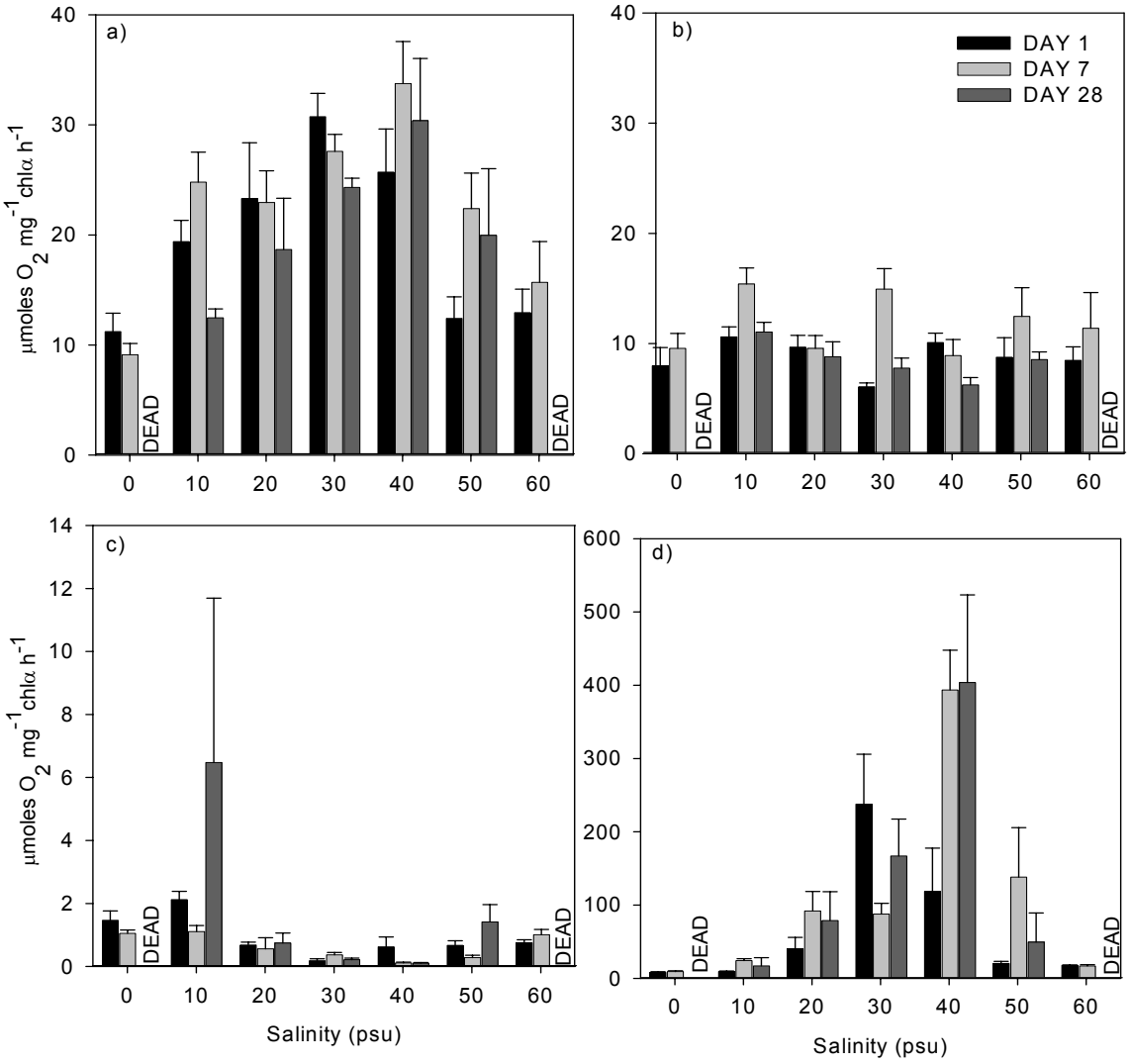

Figure 6. *Thalassia testudinum* photosynthetic responses (mean ± standard error) to experimental salinities 0 – 60 psu at exposure times of 1, 7, and 28 days; the photosynthetic parameters measured were a) Pmax, b) respiration, c) alpha, and d) I_k.

A two-way ANOVA showed both salinity and day to have significant effects on osmolality, with there being a significant interaction between the two factors (Table 2). However, the F value for salinity variability is an order of magnitude higher than that for time variability or the interaction term, indicating that salinity

has the predominant influence on variation in osmolality values in these treatments. When compared within treatments, day 1 and 7 readings are significantly different in 0, 50 and 60 psu, and day 1 and 28 readings are significantly different in 10 and 50 psu (after 28 days 0 and 60 psu plants were dead). This indicates that after one day exposure there were no osmolality adjustments in the intermediate salinities (20 – 40 psu), but that osmolality changes were significant at the extremes (Fig. 7a). Intracellular osmolality readings were higher than intercellular, but patterns of change were similar for both osmolality readings (Fig. 7b), with increased osmolality values with increased treatment salinity.

Leaf osmolality values ranged from 550 μmol kg^{-1} (0 psu, day 7) to 3325 μmol kg^{-1} (50 psu, day 28). Again, both day and salinity variability were significant with significant interaction, and salinity had the greatest overall effect (Table 3). Osmolalities in all treatment salinities were significantly different from each other. The 20 and 30 psu treatments exhibited similar patterns with no significant intercellular osmolality change over time. When compared within treatments, day 1 and 7 readings are significantly different in 50 and 60 psu, and day 1 and 28 readings are significantly different in 10, 40, and 50 psu (after 28 days 0 and 60 psu plants were dead). This indicates that there were no major osmolality adjustments in the intermediate salinities, but that osmolality changes become significant over time at the extremes (Fig. 7a).

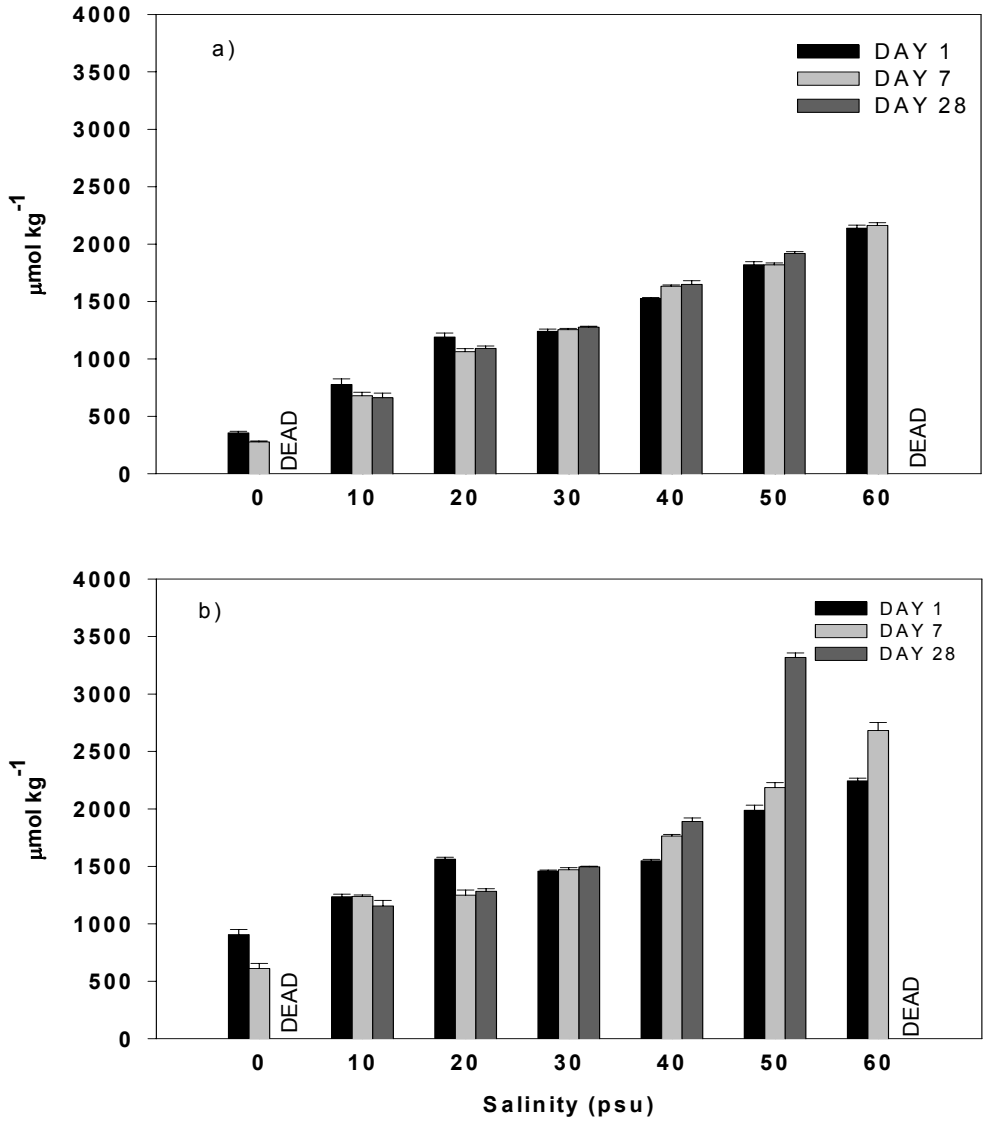

Figure 7. *Thalassia testudinum* a) intercellular and b) intracellular osmolality measurements (mean ± standard error) for plants in salinities of 0 – 60 psu taken at exposure times of 1, 7, and 28 days.

Source of variation	DF	F	P
Exposure Time	2	19.6	<0.001
Salinity	6	1809.6	<0.001
Exposure Time X Salinity	12	21.4	<0.001

Table 2. *Thalassia testudinum* two-way ANOVA comparing variation in intercellular osmolality due to salinity, exposure time, and interactions between these two factors.

Source of variation	DF	F	P
Exposure Time	2	6.2	0.004
Salinity	6	1163.5	<0.001
Exposure Time X Salinity	12	13.7	<0.001

Table 3. *Thalassia testudinum two*-way ANOVA comparing variation in intracellular osmolality due to salinity, exposure time, and interactions between these two factors.

Chapter 2: Responses of *Ruppia maritima* to salinity variations

Introduction

Ruppia maritima L.*,* widgeon grass, is a vital submerged aquatic vegetation (SAV) species in the coastal environments of the United States, and has a wider range of salinity tolerance than any other SAV species (Husband and Hickman, 1985; Kantrud, 1991; Koch and Dawes, 1991; Adams and Bate, 1994). *Ruppia* species provide food for many types of migrating waterfowl and marine organisms, as well as providing critical habitat for fish and micro-invertebrates (Congdon and McComb, 1979; Montague et al., 1989).

It has been suggested that *Ruppia maritima* is not a true seagrass. Thayer et al. (1975) define true seagrasses as angiosperms that live completely submerged in a brackish to saline medium, and carry out all of their life cycle underwater. Due to its cosmopolitan distribution in a wide variety of salinities, *R. maritima*'s classification as a seagrass has come under scrutiny. The fact that *R. maritima* is not limited to saline environments (Higgonson, 1965; Mitchell, 1979) causes some researchers to consider it to be a freshwater species due to its

tolerance to non-marine conditions (Thorne, 1954). Other aquatic plants are able to tolerate a slightly saline environment, but are not classified as seagrasses; *Potamogeton pectinatus,* for example*,* is a freshwater species, but can often be found growing in brackish water (Barbour, 1970). Other researchers maintain that *R. maritima* is not a seagrass because it does not reproduce like other seagrasses via hydrophilous pollination and submerged flowers, but instead exhibits hydroanemophilous pollination and flowers at the surface of the water (Zieman, 1982). *Ruppia* species are easily outcompeted by other SAV species in euryhaline conditions (McRoy and McMillan, 1977; Iverson and Bittaker, 1986; Jagles and Barnabas, 1989), can have a transient, weedy existence, and are often considered “disturbance” species. Neither Zieman (1982), Phillips (1960), nor den Hartog (1967) consider *R. maritima* a true seagrass, yet it grows, flowers and produces seeds at 60 psu.

Others have conflicting opinions. Several investigators refer to *Ruppia maritima* as a true seagrass species (Iverson and Bittaker, 1985; Jagles and Barnabas, 1989; Dawes et al., 1995; Murphy et al., 2003). According to a study by Lazar and Dawes (1991), even though *Ruppia* species can survive in fresh water situations, optimal growth and reproductive success occur in saline

conditions. Husband and Hickman (1985) claim saline conditions are required by *R. maritima* and not just tolerated. Studies with both wild *R. maritima* plants and those grown in culture show optimal growth occurs at salinities between 0 and 31psu (McRoy and McMillan, 1977; Thursby, 1984; Bird et al., 1993;). In lower salinity regimes, *R. maritima* is outcompeted by freshwater species (Verhoven, 1975; Howard-Williams and Liptrot, 1980; Verhoven, 1980).

Different *Ruppia* species have been placed in three different families (Kantrud, 1991), so its taxonomic classification is also open to dispute (Congdon and McComb, 1979). Due to genetic differences between the many species of *Ruppia*, physiological responses to environmental variables also differ. Within subpopulations of a *Ruppia* species, great levels of variability occur in physiological responses to salinity levels in the environment (Koch and Dawes, 1991). Others have studied the taxonomy of *Ruppia maritima*, and have come to differing conclusions as to how it should be classified (Aston, 1973; Richardson, 1980). To avoid confusion, some investigators simply refer to *R. maritima* as a submerged halophyte or macrophyte (Dunton, 1990; Adams and Bate, 1994), or refer to it only by its species name and avoid the use of the seagrass classification altogether.

In general, *Ruppia maritima* is said to be a euryhaline species and has broad tolerances to salinity (Verhoeven, 1979). *Ruppia* species are found in environments ranging from fresh water (Richardson, 1980; Wetzel and Penhale, 1983) to hyper-saline lagoons with salinities upwards of 120 psu (Simmons, 1957; McMillan and Mosely, 1967; Congdon and McComb, 1981). Given this broad range of salinity tolerance, the specific effects of salinity on various *Ruppia* species (McMillan and Mosely, 1967; Brock, 1981; Husband and Hickman, 1985; Lazar and Dawes, 1991; Adams and Bate, 1994), and *Ruppia maritima* in particular (Bourn, 1935; Mayer and Iow, 1970, Dunton, 1990; Lazar and Dawes, 1991; Bird et al., 1993) have been widely studied.

The present study was designed to determine the physiological and physical responses of *Ruppia maritima* to different salinities ranging from 0 to 60 psu in a controlled environment. The response variables that were used to determine the "stress" in seagrass associated with each salinity level included visual estimates of leaf color change from green to brown, which is evidence of tissue death, changes in plant growth and leaf turnover rates, changes in photosynthetic responses, and osmolality changes within plant blades. These variables were used to determine upper and lower salinity tolerance thresholds for *R. maritima* under laboratory conditions, and to assess the amount of "stress"

associated with each experimental salinity level between 0 and 60 psu over 1 - 28 days exposure time.

Materials and methods:

Ruppia maritima plants were collected in Madeira Bay, a section of Florida Bay, in November 1995 (Fig. 1). After collection and sterilization (Fig. 8) the plants were maintained in axenic culture in a media composed of ½ strength Murashige and Skoog Basal Salt Mixture, 1% sucrose, 10 mg/L 2iP (a cytokinin), and MES (a pH buffer) at 20 psu. Plants were subdivided monthly to generate clonal lines. Axenic clonal propagation insured near identical growing conditions for the replicates prior to experimentation, as well as insuring genetic consistency among the plants.

Each color-designated replicate came from a different parent plant, which was subdivided prior to experimentation (all white plants were clonally propagated from one parent plant). When the plants had completed a four-week growth cycle in the media, they were rinsed thoroughly in Instant Ocean © brand synthetic seawater (IO) at 20 psu and transferred to a 115 L (30 gal) aquarium filled with IO at 20 psu (Alistock et al., 1991). Once the *R. maritima* plants became autotrophic (were “weaned” from the culture medium), and had established roots, they were planted in 3x3x3’ peat pots filled with locally-collected natural sediment from Lassing Park, St. Petersburg, Florida.

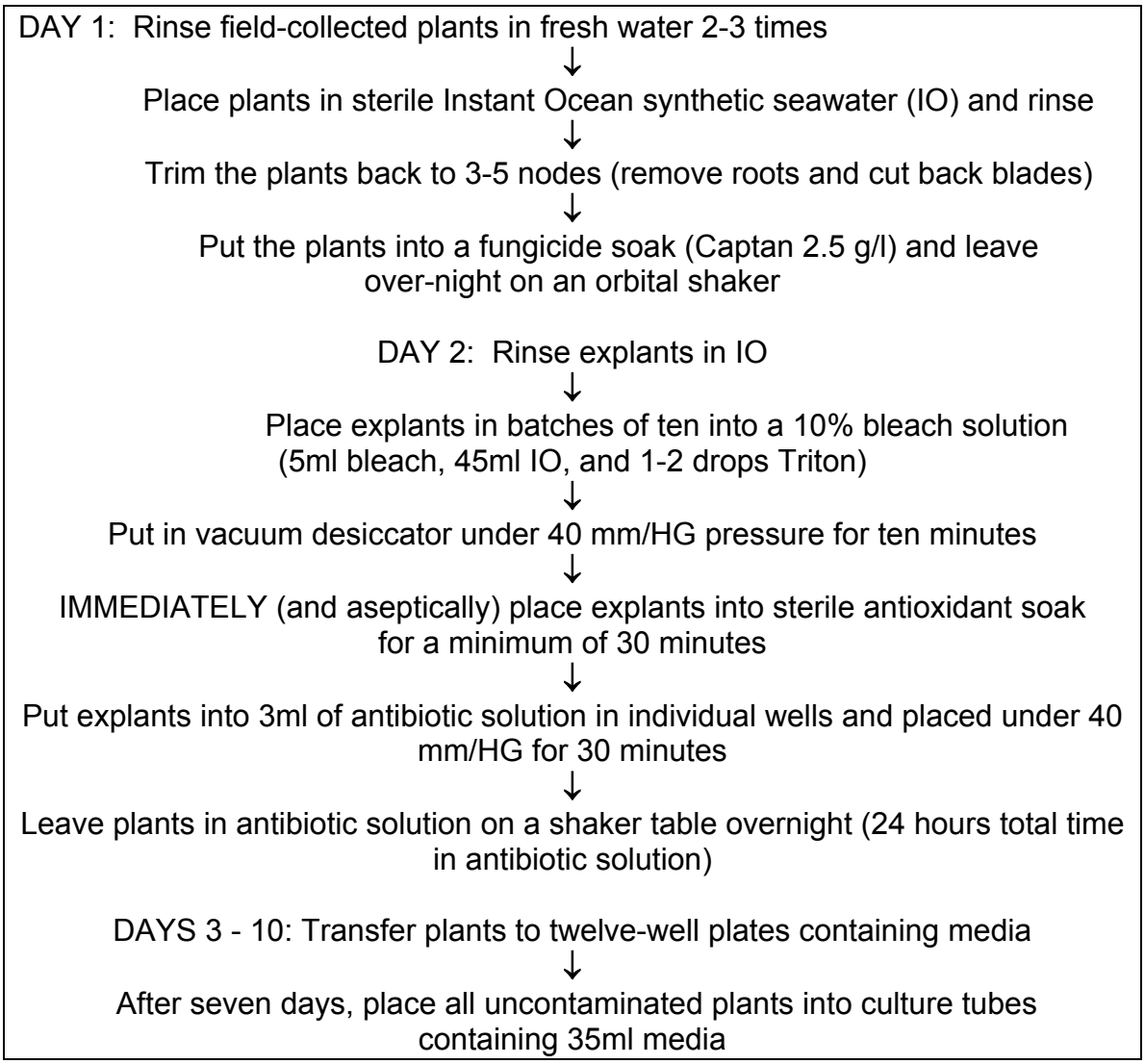

Fig. 8. Sterilization protocol for *Ruppia maritima* plants to be maintained in axenic culture (modified from Koch and Durako, 1991).

Experiments were begun after the *R. maritima* plants were established in the sediment and were growing at a steady rate. There were four plants per treatment at each salinity level. The four experimental units were identified by colored flags to allow for repeated measures analyses.

The salinity tolerance series for *Ruppia maritima* was performed as follows: all replicate plants were "reused" in a repeated measures system where

a group of blades were sacrificed for each day of experimentation. This allowed the same plant to be used throughout the 28-day treatment. At each salinity level, the experimental tanks contained eight *R. maritima* plants. Plant responses to salinity levels were monitored through measurements of visual analysis of tissue death, plant growth, photosynthesis (P) vs. irradiance (E) responses, and measurement of osmolality in leaf tissues of four replicate plants. These response variables were measured at exposure times of t = one, seven, and 28 days in each test salinity.

The plants used in the salinity-tolerance-range experiments were haphazardly chosen from a random arrangement of holding aquaria, removed from their growth salinity (20 psu), and acclimated in 10 psu increments per week from their original salinity to the various treatment salinities. Tanks were set up at salinities of 0, 10, 20, 30, 40, 50, and 60 psu. Air stones provided aeration in the tanks and tap water additions were made to counteract evaporative losses as necessary.

Once the desired salinities were reached, the individual plants were evaluated initially, and then monitored weekly for leaf tissue death as observed by blade color change (Table 1). *Ruppia maritima* growth was measured by counting new nodes and blades, measuring blade length, and weighing new plant material that developed in the treatment salinities. The plants were marked

with aluminum markers two nodes back from the end of a branch (t = 7). After 14 days, all material (new growth) past these two nodes on the branch was harvested (t = 28). The number of new nodes and blades were counted, and the blades were measured; the material was dried at 60°C to a constant weight (72 hrs) and weighed to determine the amount of growth that occurred.

In the experiments to measure the physiological responses of *Ruppia maritima* to salinity, eight blades were randomly removed from each replicate, and a total of four and a half cm from each blade was used. The first 2 cm from the base of the blades was used in P vs. E measurements. Forty mm segments from five of these blades were used in osmolality determination, and two cm segments were cut from all eighth blades to be used for dry weight measurements. After the P vs. E measurements were completed, the leaf material used was frozen and processed later for chlorophyll extraction with acetone.

Photosynthesis was measured as a change in concentration of dissolved oxygen in a closed system, as outlined by Beer et al. (1977) and Durako and Kuss (1994). A Hansatech DW/1 Clark-type oxygen-electrode system was used to measure Photosynthesis (P) vs. Irradiance (E) responses for the four

replicate plants in each salinity treatment. The plant material was placed in a closed chamber filled with 2.5 ml of N_2 sparged seawater with the appropriate test salinity. A magnetic stirring bar inside the chamber provided vigorous stirring within the chamber. The temperature in the chamber was controlled by water from a 25° C water bath being circulated through the outer jacket of the chamber. Light was provided by a Kodak ectographic slide projector with a 300-watt bulb. Light intensities were varied by placing neutral density filters between the projector and the plant chamber of the experimental set up. Light levels were measured with a cosine-corrected quantum sensor connected to a Li Cor datalogger (model LI-1000).

After the plant material was placed in the chamber with 2.5 ml Instant Ocean, it was allowed to equilibrate in the dark for ten minutes. After equilibration, the plants were subjected to twelve light levels increasing from ≈10uE to ≈1000uE of photosynthetically active radiation (PAR = 400 - 700nm). There was a one-minute equilibration time at each light level before the initial O_2 reading was made. Another reading was taken two minutes later, and the resulting del (Δ) value was used for oxygen-flux calculations.

Photosynthesis and respiration are expressed in μmoles O_2 mg^{-1} chl*a* h^{-1}.

All Hansatech readings are net photosynthesis, with respiration being the initial dark reading. The calculated P vs. E response variables were alpha; the initial slope of the regression line, Pmax; the light level at which maximum photosynthetic activity was reached, and I_k, which is called the saturation irradiance, and is calculated is Pmax/alpha. Respiration was the initial two-minute Δ value recorded after the ten-minute dark-incubation period. The response variables were calculated using a least squares nonlinear curve-fitting algorithm in Sigma Stat (Jandel Scientific, CA). The P vs. E data were fit to the hyperbolic tangent equation (y = [p*tanh (a*x/p)]+r), as described by Jassby and Platt (1976), where p = Pmax and a = alpha. All P vs. E curves were plotted as gross photosynthesis and as a function of the curve fit (Fig. 9). These curve-fitting equations were used because the *Ruppia maritima* P vs. E curves exhibited typical saturation kinetics similar to those described by Jassby and Platt.

Osmolality was measured using a Wescor Vapor Pressure Osmometer 5500C. A modified version of the leaf disc method proposed by Tyerman (1982, method 1) was used. The osmometer was calibrated against two standards, 290 and 1000 mmol/kg, to encompass the full range of possible readings.

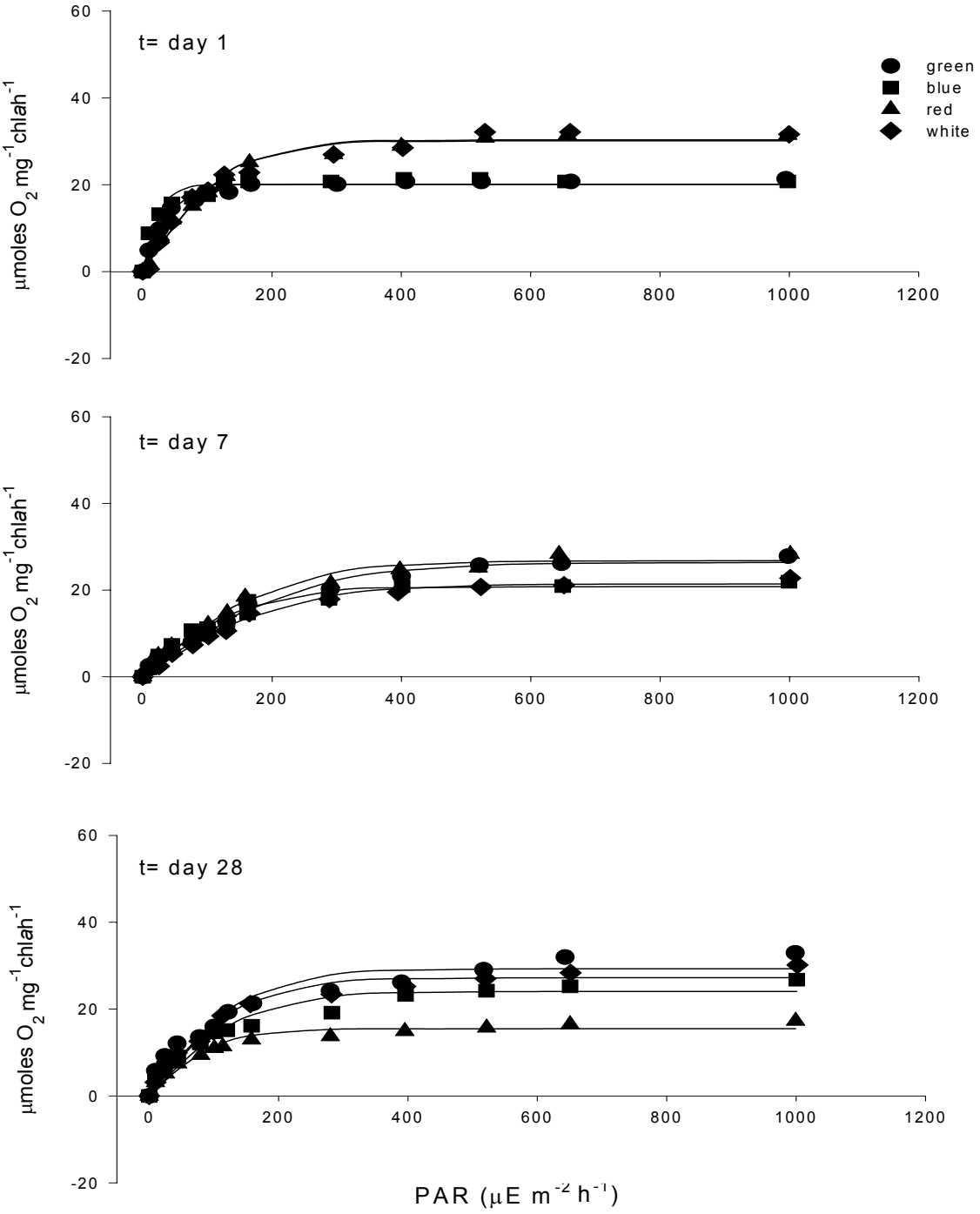

Fig. 9. Graph showing gross photosynthesis (scatter plots) of *Ruppia maritima* plants in 20 psu Instant Ocean synthetic seawater at exposure times of 1,7, and 28 days. The data are also shown as a function of the hyperbolic tangent equation y = Pmax*tanh (alpha*x/Pmax) (spline curves).

Five 4 mm *Ruppia maritima* blade segments were used for each osmolality determination. The blades were cut while submerged, the tissue was quickly blotted to remove any surface water, and the tissue was immediately placed in the osmometer thermocouple chamber. The plant tissue was allowed to equilibrate inside the thermocouple for 20 minutes before a reading was made. This initial reading was a measure of leaf water potential, or intercellular osmolality. The tissue was then frozen to fracture internal membranes, and a second osmolality reading was taken. Due to the delicate nature of the *R. maritima* segments, they were frozen for only two hours; an extended freezing time increased readings to unrealistically high levels, most likely due to desiccation. This second reading was a measure of the intercellular osmotic pressure, and measures total ion concentrations.

Results

Changes in leaf color

Leaf color changes were most extreme in 60 psu, with some of the replicates dying by day 28. There was less leaf discoloration in plants in 0, 10, and 50 psu treatments. No color change was detected in 20, 30, or 40 psu treatments (Fig. 10).

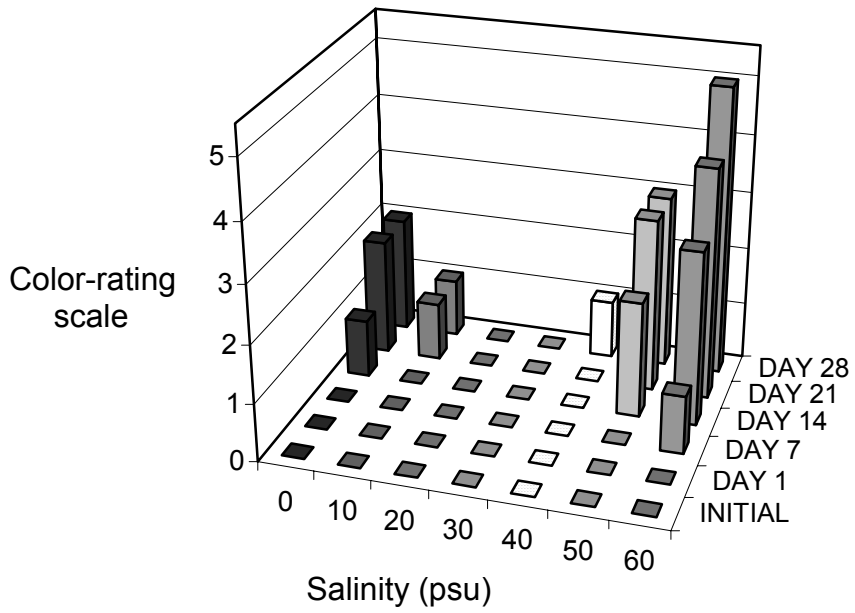

Fig. 10. *Ruppia maritima leaf* color change observed weekly over the 28-day experimental period in salinities 0 – 60 psu. Color scores are based on a 0 - 5 rating scale, with 0 being 100% green and 5 being 100% brown.

Changes in leaf growth rates

Maximum growth rates of *Ruppia maritima*, as measured by leaf area, weight, and leaves and nodes produced per day, occurred at 20 psu (ambient) which was significantly different from those in all other treatment salinities. Leaf area growth rates ranged from 0.25 cm day^{-1} (60 psu) to 4.5 cm day^{-1} (20 psu) (Figs. 13a, b, c, d). All growth parameters decreased significantly as the treatment salinities were varied from 20 psu (growth salinity); however, higher rates for all growth parameters were recorded in salinities 30 psu and lower, as opposed to 40 – 60 psu.

Changes in photosynthetic characteristics

Salinity significantly affected the Pmax values in *Ruppia maritima.* Exposure time had no significant effect by itself, but there was significant interaction between salinity and exposure time. F values were similar for all three sources of variation, indicating all three had similar effects on salinity (Table 4). The differences occurred at the extreme salinities at maximum exposure time (Fig. 12a). This indicates that the plants are able to adjust to most salinities over time, but are still compromised at the upper extremes.

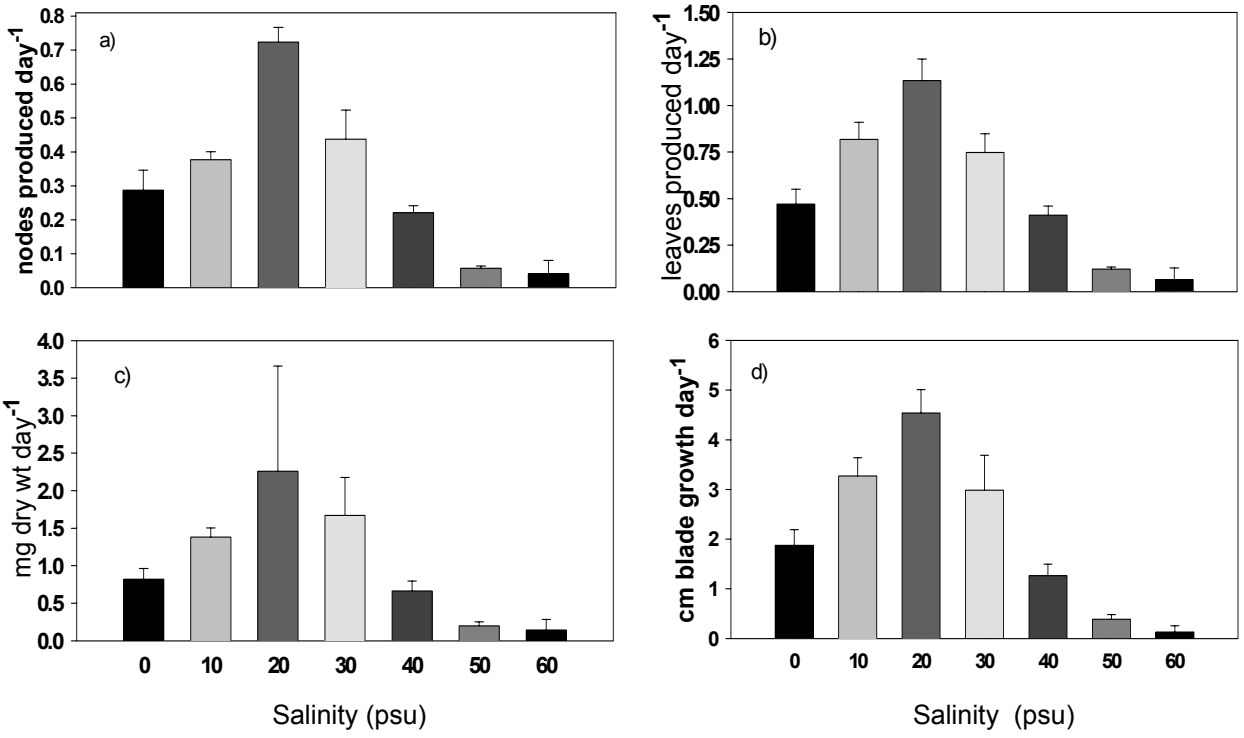

Fig. 11. Mean *Ruppia maritima* growth rates (mean ± standard error) from exposure time t = 7 days to t = 28 days in experimental salinities of 0 – 60 psu.

Respiration, I_k, and alpha showed similar responses, with salinity, exposure time, and the interaction between these two having significant effects. As with Pmax,

respiration and alpha had similar F values (Table 5 and 6), but I_k had a higher F for salinity driven responses (Table 7). Respiration and I_k values fluctuated during the treatment period, but there was no significant change over time (day 1 vs. 28), suggesting some level of adaptation to the new salinity.

Source of variation	DF	F	P
Exposure Time	2	2.4	0.098
Salinity	6	2.3	0.047
Exposure Time X Salinity	12	2.4	0.011

Table 4. *Ruppia maritima* two-way ANOVA comparing variation in Pmax values for exposure times of 1,7, and 28 days in treatment salinities 0 – 60 psu.

Source of variation	DF	F	P
Exposure Time	2	3.4	0.040
Salinity	6	3.9	0.002
Exposure Time X Salinity	12	2.8	0.004

Table 5. *Ruppia maritima* two-way ANOVA comparing variation in respiration values for exposure times of 1,7, and 28 days in treatment salinities 0 – 60 psu.

Source of variation	DF	F	P
Exposure time	2	7.4	0.001
Salinity	6	7.1	<0.001
Exposure Time X Salinity	12	6.4	<0.001

Table 6. *Ruppia maritima* two-way ANOVA comparing variation in alpha values for exposure times of 1,7, and 28 days in treatment salinities 0 – 60 psu.

Source of variation	DF	F	P
Exposure time	2	9.1	<0.001
Salinity	6	25.2	<0.001
Exposure Time X Salinity	12	6.0	<0.001

Table 7. *Ruppia maritima two*-way ANOVA comparing variation in I_k values for exposure times of 1,7, and 28 days in treatment salinities 0 – 60 psu.

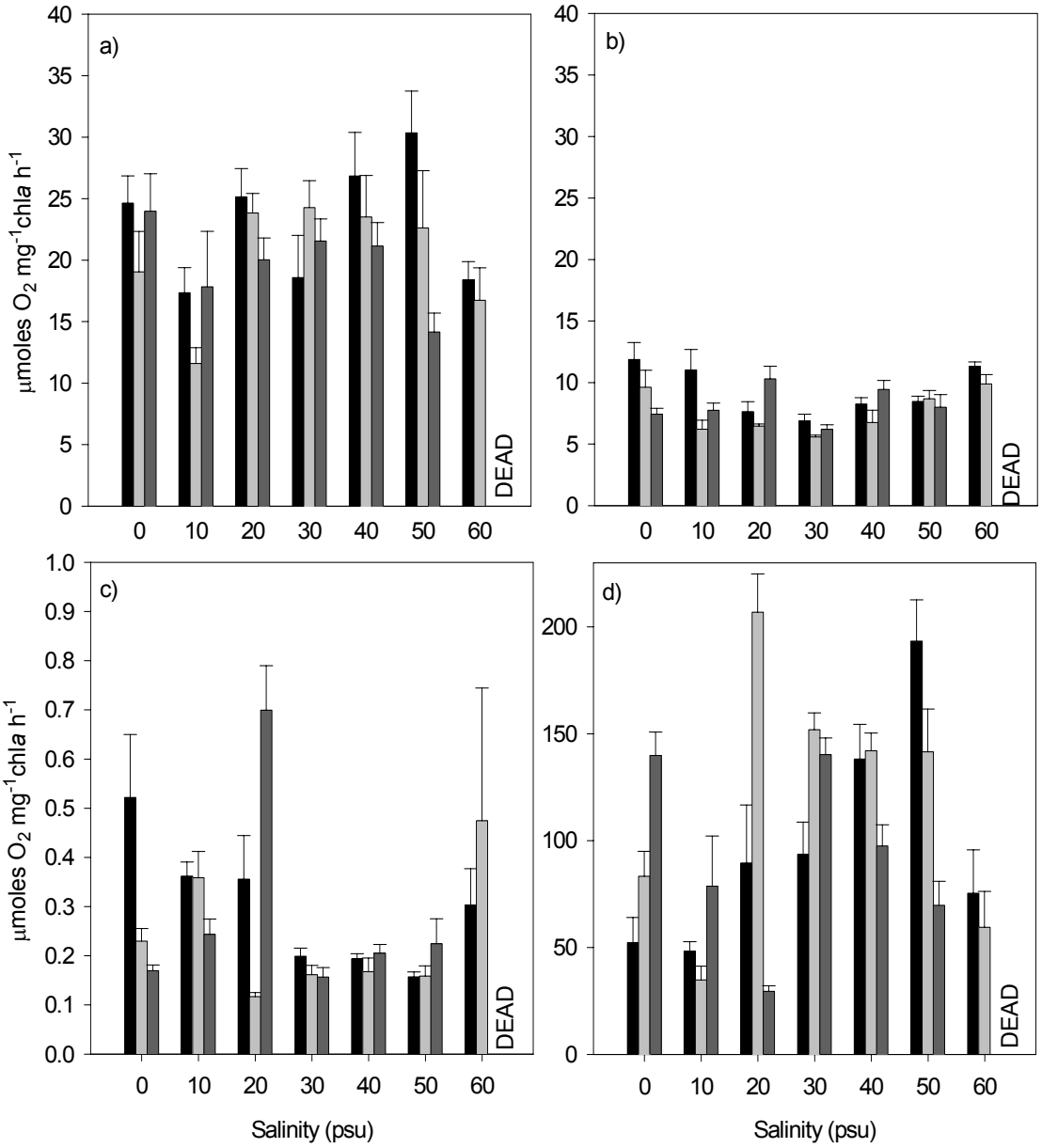

Fig. 12. Photosynthetic responses of *Ruppia maritima* (mean ± standard error) to different salinities ranging from 0 to 60 psu at exposure times of 1, 7, and 28 days. The parameters examined were a) Pmax, b) respiration, c) alpha, and d) I_k.

Respiration and alpha values for 60 psu are significantly different than the other salinity levels (Fig. 12b and c), and I_k values in the 20 - 50 range were significantly different from those at the extremes (Fig. 12d).

Changes in leaf tissue osmolality

Both intercellular (fresh) and intracellular (frozen) osmolality values followed the same pattern, increasing with increasing salinity of treatment medium (Figs.13 a and b). Intercellular osmolality readings were closer to those of the treatment media in which the plants were growing than were the intracellular readings were (Table 8).

PSU	treatment media	intercellular Δ	intracellular Δ
0	15.3±0 .9	295	359
10	341.5±1.3	365	481
20	630.0±1.6	298	355
30	898.6±1.5	362	390
40	1212.4±1.3	329	372
50	1550.2±3.3	307	398
60	1788.6±1.2	338	660

Table 8. Osmolality values in μmoles kg^{-1} for the treatment media ranging from 0 to 60 psu and the Δ values for intercellular and intracellular osmolality of *Ruppia maritima* at exposure time t = 1 day in these treatment salinities.

Intercellular osmolality was significantly affected by both exposure time and salinity, and there was a significant interaction between the two. Osmolality readings ranged from a low of 150 μmoles kg^{-1}in 0 psu on day 28 to a high of 2450 μmoles kg^{-1} in 60 psu on day 7. Osmolalities in all treatment salinities were significantly different from each other, except for 20 psu vs. 10 psu on day 7. The 0, 20, 30, and 40 psu treatments exhibited similar patterns with no significant intercellular osmolality change over time. Within salinities, only the 50 psu treatment exhibited significant change over exposure time, between the day 1 and day 7. The day 1 and day 28, however, were significantly different in 0, 10,

and 50 psu treatments, with replicates in 60 psu dying by this time. These data again indicate more “stress” response at the extremes.

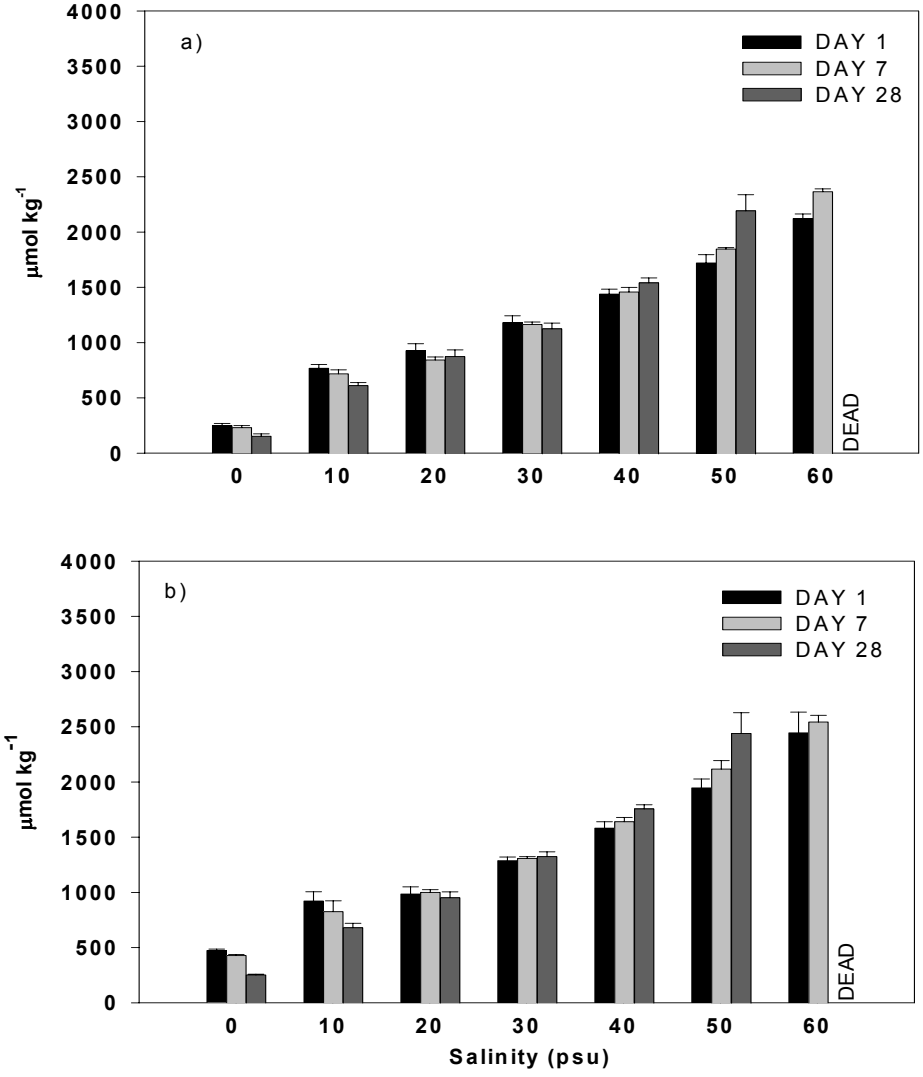

Fig 13. *Ruppia maritima* a) intercellular and b) intracellular osmolality (mean ± standard error) for plants in salinities of 0 – 60 psu taken at exposure times of 1, 7, and 28 days.

Intracellular osmolality was significantly affected by salinity and the interaction between exposure time and salinity, but time was not a significant main effect. However, the large differences in F values between the variables

allow one to infer that salinity had the major physiological influence on osmolality measurement variations (Table 9 and 10).

Intracellular osmolality readings ranged from a low of 250 in 0 psu on day 28 and a high of 2600 in 60 psu on day 7. Within all salinity levels, day 1 was significantly different from day 28, and the day 1 and day 7 were only significantly different in salinities of 10 and 50 psu. There was a significant difference between day 1 and 7 in 60 psu, but by day 28, the 60 psu replicates were dead. Intracellular osmolality readings had the same pattern of increase and decrease with salinity, but these changes were significant over exposure time in all treatments except 20 and 30 psu.

Source of variation	DF	F	P
Exposure Time	2	4.3	0.018
Salinity	6	972.2	<0.001
Exposure Time X Salinity	12	9.0	<0.001

Table 9. *Ruppia maritima* two-way ANOVA comparing variation in intercellular osmolality values due to salinity, exposure time, and interactions between these two factors.

Source of variation	DF	F	P
Exposure time	2	1.6	0.219
Salinity	6	693.7	<0.001
Exposure time X Salinity	12	7.1	<0.001

Table 10. *Ruppia maritima* two-way ANOVA comparing variation in intracellular osmolality values due to salinity, exposure time, and interactions between these two factors.

Chapter 3: Synthesis of Physiological Responses of *Thalassia testudinum* and *Ruppia maritima* to different salinity levels

The physiological responses of *Thalassia testudinum* and *Ruppia maritima* to different salinity levels indicate that significant increases and decreases in growth salinity are initially stressful to both species. *R. maritima* has a greater ability to adjust to new, especially reduced salinities that *T. testudinum* lacks. There was an optimal salinity level for both species as well as a range of salinities in which the long-term physiological stresses did not cause tissue death. For *T. testudinum*, the optimal salinity was 40 psu, as seen in highest Pmax and growth rates. This optimal level was 10 psu above the growth salinity. For *R. maritima,* the optimal salinity was 20 psu, the growth salinity in which *R. maritima* exhibited highest growth rates, fewest osmotic adjustments, and no discoloration.

Overall, it is clear that in this study *T. testudinum* shows fewer stress responses to salinities 20, 30, and 40 psu and that *R. maritima* exhibits less stress response at lower salinities (0 - 40 psu), where there was less leaf discoloration, growth rates were more rapid, and osmolality readings were less varied over time, and Pmax was elevated. Neither seagrass species did well in 60 psu, with replicates dying by day 28. *T. testudinum* physiological response variability was driven by salinity differences, and not dependant on exposure

time. In contrast, most *R. maritima physiological* response variability is dependant on salinity, exposure time, and the interaction between these two variables.

Changes in leaf color

Thalassia testudinum showed a color-change response to alterations in salinity. Plants in the 20 - 40 psu range showed no color change. In other treatment salinities browning greatly increased, with plants in 0 and 60 becoming 100% brown (dead) by day 28. *Ruppia maritima* also showed a color-change response to different salinity levels. Twenty and 30 psu elicited no color change over time. Plants in 0, 10 and 40 psu showed slight browning as exposure time increased. Plants in 50 and 60 psu treatments were up to 50 and 100% brown (dead) respectively by day 28. Based on leaf color, both species show an optimal range in which there is no substantial (<25%) discoloration, 20 – 40 for *T. testudinum* and <40 for *R. maritima*. Significant changes in *T. testudinum* blade color were seen in the extreme salinity treatments (0, 10, 50, and 60 psu), and in the upper salinity extreme (50 and 60) *for R. maritima*. However, T*. testudinum* tissue death (100% brown) only occurred in the 0 and 60 psu treatments after 28 days, showing short-term tolerance to salinities of 0 and 60 psu, and a greater tolerance for 10 and 50 psu (for at least up to 28 days exposure time). Sculthorpe (1967) also observed short-term survival of *T. testudinum* in 3.5 psu, and McMillan and Moseley (1967) observed some survival at 60 psu. *R. maritima* only died in the 60 psu treatment, showing lower tolerance for this salinity level.

Changes in leaf growth rates

For all growth parameters measured for *Thalassia testudinum,* 20, 30, and 40 psu treatments were the least stressful for the plants. Forty psu appears to be the optimal treatment salinity, based on highest growth rates. There was short-term tolerance for 10 and 50 psu treatments, but none for 0 or 60 treatment salinities. *Ruppia maritima* growth values were significantly higher in 20 psu (growth salinity), and higher values for all growth parameters were seen in salinities less than 40 psu, showing fewer stress responses in lower salinities. *Ruppia maritima suffered* less stress, and maximum growth occurred in the salinity in which the plants were originally grown; this was also reported by Teo et al. (2001), with *R. maritima* grown in 10 psu.

Changes in photosynthetic characteristics

Salinity effects on photosynthesis were less pronounced in *Ruppia maritima* than in *Thalassia testudinum*, which would be expected when comparing a euryhaline species to a more stenohaline species. The Pmax for both species was somewhat affected by salinity changes, but the plants did not appear to be photosynthetically compromised in their “optimal” ranges over time.

Thalassia testudinum exhibited a salinity response in all photosynthetic parameters except respiration in all treatment salinities, with the least effect being seen in 30 and 40 psu. Significant changes in *T. testudinum* blade photosynthesis were seen in the extreme salinity treatments (0, 10, 50, and 60 psu). Forty psu appears to be the optimal treatment salinity for *T. testudinum* based on highest Pmax values. Due to repeated measures, leaf 2 was used for

experimentation on day 7. The number two leaf has been shown to be the most photosynthetically active tissue (Durako and Kuntzelman, 2002), and spikes seen in day 7 values could be due to a difference in leaf rank of sample tissue.

Thalassia testudinum respiration was not effected by salinity or exposure time. Other research has shown that salinity differences apparently have varying results on respiration rates of seagrasses. In *Zostra,* Beibl and McRoy (1971) observed an increase in respiration with increased salinity, Ogata and Takada (1968) recorded a decrease in respiration over the same range, and Kerr and Strother (1985) found no significant respiration response at all. In *Halophila johnsonii* the lowest salinity effect (F value) is observed in respiration compared to Pmax, alpha, and I_k (personal communication Durako, 2003). The lack of significant variability in respiration rates among the salinity treatments was surprising, and indicates that respiration is not a useful response variable for determining physiological stress in these seagrass species.

Ruppia maritima exhibited a response to all measured photosynthetic parameters in all treatment salinities. For most parameters, exposure time, salinity, and the interaction between these two variables were all significant, and therefore, significance in the variability of the photosynthetic responses was difficult to ascertain. Most significant changes were seen in 60 psu, which these data demonstrate to be the most "stressful" salinity on *R. maritima*.

Changes in leaf tissue osmolality

Both *Thalassia testudinum* and *Ruppia maritima* show that variability in osmolality values is dependent upon salinity, exposure time, and the interaction

between these two variables. For both intercellular and intracellular osmolality, the salinity F values were at least 100 times that of exposure time or interaction, so salinity was the major influence on osmolality variability for both species. The trend in osmolality shows increases or decreases when leaves are removed from the growth salinity level. Meyer et al. (1989) and Tyerman et al. (1984) observed a pattern of osmolality increasing with elevated salinities due to an ability of SAV to stabilize their osmotic potential by increases in internal ions. Most of the change in osmolality appeared to occur by the first day, except in treatments outside of the optimal ranges, in which the plants were rapidly becoming dysfunctional. For *R. maritima*, these changes only became significant over the entire duration of the experiment at the highest salinity, showing a stress response at the upper extreme over time. *Thalassia testudinum* osmolality values varied over time in both the upper and lower extremes, showing that outside of the optimal range, *T. testudinum* becomes compromised.

These osmolality data indicate that for both species, the plants within their optimal range are making initial internal adjustments quickly, and for both species, plants outside the optimal range continue making adjustments over time to deal with the change in salinity**.** This stabilization in the 20 - 40 psu range for *Thalassia testudinum,* and <40 psu for *R. maritima* agrees with the conclusion of Meyer et al. (1989) that there is no change in leaf tissue osmolality with time if the plants are not compromised. The intracellular adjustments in osmolality over longer time periods are most likely due to an increase of accumulated organic solutes, such as proline, in the cell cytoplasm (Brock, 1981; Wyn Jones and

Gorham, 1983; Van Digglen et al., 1987). This was seen in *Ruppia maritima*, with an immediate osmolality change occurring with exposure to a new salinity, and a second change occurring after 1 to 2 days in the treatment, that was accompanied by an increase in internal solutes (Murphy et al., 2003).

Considering the vital role seagrasses play in the nearshore marine environment and the recent die off of these important species in Florida Bay (Robblee et al., 1991), one can logically argue for the need to limit anthropogenic influences on fresh water flow into coastal regions. Humans can impact the coastal marine environment through control of freshwater discharges, which result in salinity fluctuations. Weekly fluctuations from high to low salinities negatively impact *Ruppia maritima* when compared to moderate fluctuations around 20 psu (Wimmers, 1998). *Thalassia testudinum* plants exhibited stronger stress responses than *R. maritima* to fluctuating salinity, including defoliation and impaired osmoregulation (Chesnes, 2001). The results of my experiments on the physiological responses of *T. testudinum* and *R. maritima* indicate that water management practices benefiting both species would 1) maintain salinities between 30 and 40 psu for *T. testudinum* and 10 to 30 psu for *R. maritima and* 2) maintain salinity levels at 40 psu or less at all times for both species.

Salinity fluctuations elicit stress responses in seagrasses although salinity changes alone may not cause seagrass mortality. These stress responses may contribute to the decline of grass beds already under other environmental pressures. Recent studies of the pathogen *Labyrinthula* in *Thalassia testudinum*

in Florida Bay indicate that stressed seagrass is negatively impacted by *Labyrinthula* while "healthy" seagrass is not (Blakesley et al. a, in prep). Those studies also showed that low salinities inhibit *Labyrinthula* infection of *T. testudinum*. However, stable mid-range salinity levels (20 – 40 psu), good for both *T. testudinum* and *R. maritima,* would promote *Labyrinthula* infection and spread of disease in dense *Thalassia* beds (Blakesley et al. b, in prep). However, lower salinity pulses that can be tolerated by *T*. *testudinum*, but not *Labyrinthula,* could keep infections levels to a minimum.

Due to the importance of seagrass beds in the marine environment, their destruction may start a chain reaction that affects the marine organisms that depend directly upon the beds (Butler et al., 1995), and eventually the humans who depend upon these marine resources. However, if proposed changes to water management in Southwest Florida are implemented and freshwater-flow to Florida Bay is greatly increased, changes in the composition of seagrass beds are predicted for the area (Fourqurean et al., 2003). A lowering of mean salinity could favor *Halodule writii* growth over *Thalassia testudinum* growth (Lirman and Cropper, 2003). In addition, increasing fluctuations as well as decreased mean salinity would allow expansion of *Ruppia maritima* beds. Most likely, any species composition change due to increased freshwater will be affected by other water quality parameters besides just salinity (Tomasko and Hall, 1999), and will result in changing parts of Florida Bay from a clear-water *Thalassia testudinum dominated* system to a more turbid-water, mixed-species system.

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Appendices

Appendix A: Photosynthesis vs. Irradiance Data Sheet

PHOTOSYNTHESIS VERSUS IRRADIANCE DATA SHEET								
Tissue:								
Date:		Notes:						
Temperature:		Salinity:		Air Saturation:			μM/mV:	
Slide	Filter	PFD	T_1	mV	T_{1+2}	mV	Δ	
Respiration			1	3				
1	4		4	6				
2	4		7	9				
2	3		10	12				
2	2		13	15				
3	2		16	18				
4	2		19	21				
5	2		22	24				
5	1		25	27				
4	0		28	30				
5	0		31	33				
6	0		34	36				
7	0		37	39				
Tissue:								
Date:		Notes:						
Temperature:		Salinity:		Air Saturation:			μM/mV:	
Slide	Filter	PFD	T_1	mV	T_{1+2}	mV	Δ	
Respiration			1		3			
1	4		4		6			
2	4		7		9			
2	3		10		12			
2	2		13		15			
3	2		16		18			
4	2		19		21			
5	2		22		24			
5	1		25		27			
4	0		28		30			
5	0		31		33			
6	0		34		36			
7	0		37		39			

Appendix B: Chlorophyll Analysis Data Sheet

P vs. E chlorophyll and dry weight data							
Pot #	Days	Treatment	Rep	Sub	ID	extract	DWT

Appendix C: *Thalassia testudinum* Growth Data

Thalassia testudinum growth		Plants marked on day 7, harvested on day 21.		
Salinity	Replicate	Production (mg/day)	Turnover (days)	Leaf Area Production (cm^2 /day)
0	1	0	0	0
0	2a	0.0357	372.4	0.00714
0	2b	0.05	194	0.0107
0	3	0	0	0
10	1a	0.347	88.753	0.086
10	1b	0.611	78.521	0.093
10	2a	0.563	87.766	0.114
10	2b	0.444	58.119	0.086
10	3	0.704	71.761	0.139
20	1	0.831	74.791	0.111
20	2a	0.508	118.163	0.136
20	2b	0.974	68.04	0.286
20	3a	0.822	61.623	0.304
20	3b	0.877	36.815	0.261
30	1a	0.967	50.634	0.354
30	1b	0.857	42	0.209
30	2a	1.123	51.791	0.261
30	2b	1.164	51.791	0.261
30	3a	1.286	42.194	0.232
30	3b	1.429	56	0.229
40	1a	0.926	50.588	0.254
40	1b	1.154	44.809	0.307
40	2	1.33	41.729	0.364
40	3	2	39.54	0.539
50	1a	0.686	66.85	0.189
50	1b	0.759	76.151	0.154
50	2	0.84	48.726	0.325
50	3a	0.686	139.199	0.163
50	3b	0.531	77.79	0.154
60	1	0.149	143.282	0.034
60	2a	0.161	129.796	0.026
60	2b	0.211	148.703	0.096
60	3	0.239	177.347	0.025

Appendix D: *Ruppia maritima* Growth Data

Plants marked on day 7, harvested on day 21. Three branches were marked on each plant.

sal	rep	nodes	leaves	dwt	branch	nodes/ rep	nodes/ day	leaves/ rep	leaves/ day	cm/day /rep	wt/ rep	dwt/day/ rep
0	1a	5	7	0.013	y	4.67	0.33	7.67	0.55	2.19	0.015	0.0010
0	1b	4	6	0.011	n							
0	1c	5	10	0.020	n							
0	2a	0	0	0.000		2.33	0.17	4.33	0.31	1.24	0.008	0.0006
0	2b	4	7	0.012	n							
0	2c	3	6	0.011	n							
0	3a	5	7	0.012	n	5.00	0.36	7.67	0.55	2.19	0.012	0.0009
0	3b	4	8	0.012	n							
0	3c	6	8	0.013	y							
10	1a	6	10	0.014	n	5.67	0.40	10.00	0.71	2.86	0.018	0.0013
10	1b	5	8	0.010	n							
10	1c	6	12	0.029	n							
10	2a	6	15	0.022	n	4.67	0.33	10.33	0.74	2.95	0.017	0.0012
10	2b	4	8	0.013	n							
10	2c	4	8	0.017	n							
10	3a	5	12	0.016	y	5.67	0.40	14.00	1.00	4.00	0.023	0.0016
10	3b	6	14	0.029	y							
10	3c	6	16	0.024	n							
20	1a	10	9	0.023	y, 2x	9.33	0.67	13.00	0.93	3.71	0.030	0.0022
20	1b	11	18	0.032	y							
20	1c	7	12	0.036	n							
20	2a	11	18	0.043	y	9.67	0.69	18.67	1.33	5.33	0.036	0.0025
20	2b	8	15	0.027	n							
20	2c	10	23	0.037	y							
20	3a	11	14	0.028	y	11.33	0.81	16.00	1.14	4.57	0.029	0.0021
20	3b	9	16	0.021	y							
20	3c	14	18	0.039	y, 2x							
30	1a	9	13	0.026	y	8.33	0.60	12.33	0.88	3.52	0.026	0.0019
30	1b	6	9	0.019	y							
30	1c	10	15	0.034	n							
30	2a	5	10	0.027	n	5.67	0.40	11.33	0.81	3.24	0.028	0.0020
30	2b	6	8	0.030	n							
30	2c	6	16	0.028	y, 2x							
30	3a	6	10	0.021	y	4.33	0.31	7.67	0.55	2.19	0.015	0.0011

Appendix D: (Continued)

30	3b	0	0	0.000								
30	3c	7	13	0.025	y							
40	1a	5	9	0.013	n	3.00	0.21	5.67	0.40	1.62	0.008	0.0006
40	1b	4	8	0.012	y							
40	1c	0	0	0.000								
40	2a	5	10	0.019	n	3.67	0.26	7.00	0.50	1.50	0.013	0.0009
40	2b	3	5	0.008	n (tiny)							
40	2c	3	6	0.011	n							
40	3a	3	6	0.011	y	2.67	0.19	4.67	0.33	0.67	0.007	0.0005
40	3b	0	0	0.000								
40	3c	5	8	0.009	n (tiny)							
50	1a	2	5	0.011	n	0.67	0.05	1.67	0.12	0.48	0.004	0.0003
50	1b	0	0	0.000								
50	1c	0	0	0.000								
50	2a	0	0	0.000		1.00	0.07	2.00	0.14	0.29	0.003	0.0002
50	2b	0	0	0.000								
50	2c	3	6	0.008	n (tiny)							
50	3a	2	4	0.006		0.67	0.05	1.33	0.10	0.38	0.002	0.0001
50	3b	0	0	0.000								
50	3c	0	0	0.000								
60	1a	0	0	0.000		0.00	0.00	0.00	0.00	0.00	0.000	0.0000
60	1b	0	0	0.000								
60	1c	0	0	0.000								
60	2a	3	5	0.010	n	1.67	0.12	2.67	0.19	0.38	0.006	0.0004
60	2b	0	0	0.000								
60	2c	2	3	0.008								
60	3a	0	0	0.000		0.00	0.00	0.00	0.00	0.00	0.000	0.0000
60	3b	0	0	0.000								
60	3c	0	0	0.000								